THE PRIMARY IMMUNE RESPONSE IN MICE

I. The Enhancement and Suppression of Hemolysin Production by a Bacterial Endotoxin*

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It is well known that macrophages of the reticuloendothelial system take up antigens before the initiation of the immune response (1) and that injections of bacterial endotoxins first depress and then stimulate the phagocytic activity of these cells (2, 3). In view of these facts, a study has been made of the influence of a *Salmonella typhosa* endotoxin on the production of specific immunoglobulins, in the hope of learning something more about the role of phagocytic cells in the process of antibody formation. A particulate antigen, the sheep red blood cell, was employed in this investigation so that changes in the uptake and processing of antigen might more readily be reflected as differences in the resulting formation of antibodies. When the endotoxin was given simultaneously with this antigen, the adjuvant effect on antibody formation previously described by others using soluble antigens (4, 5) was promptly confirmed.

On the other hand when the bacterial preparation was allowed to exert a more sustaining action and was given prior to the particulate antigen, it was found to suppress instead of enhance the production of hemolysins. This surprising effect, which in many instances resulted in the complete inhibition of antibody production, was realized by employing appropriate time intervals between the injection of endotoxin and the particulate antigen, and suitable routes of administration of these agents. These results are detailed in the present paper together with other serological findings on the effects of endotoxin on hemolysin production in mice. Subsequent to a preliminary communication of these findings (6), others (7, 8) have also reported that, under specified conditions, bacterial endotoxins will inhibit the production of antibodies to particulate antigens.

The pronounced inhibition and enhancement of antibody formation, which could be brought about at will by the proper use of endotoxin, offered an excellent opportunity to investigate the cellular changes in the antibody-forming

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lymphoid tissues of the experimental animals. These findings will be reported in the accompanying paper (9).

Materials and Methods

Mice.—Female mice of the Rockefeller Swiss Nelson-Collins strain (10) weighing 20–22 g were employed. These animals, which are highly resistant to the lethal effects of bacterial endotoxins and practically free from Gram-negative organisms (11), were housed at 72 ± 2°F, fed ad lib., and given drinking water containing 1 mg of Terramycin (soluble powder, Chas. Pfizer & Co., New York) per milliliter.

Antigen.—Suspensions of sheep red blood cells (Cappel Laboratories, West Chester, Pa.) were washed, adjusted with sterile physiological saline, and standardized in terms of hemoglobin content. When lysed with 14 volumes of 0.1% Na_2CO_3, a 1% suspension gave an optical density reading of 0.135 at 541 mμ in a Beckmann DU spectrophotometer. 0.1 ml of this preparation, containing approximately 2×10^7 cells, was employed as the standard dose of antigen.

Endotoxin.—A commercial preparation of bacterial endotoxin (Difco Laboratories, Detroit, Mich.), the lipopolysaccharide *S. typhosa* 0901 (Difco), was employed in most experiments. In addition samples of "nontoxic" and "toxic" endotoxin of *Escherichia coli* 0:113, obtained through the courtesy of Dr. Hans Noll, were also tested. The nontoxic endotoxin was the soluble product of a dioxane extraction of the bacterium (12). The toxic endotoxin was prepared by a trichloroacetic acid extraction of the residue remaining after dioxane treatment. The lipid components of these preparations were removed with chloroform methanol (2:1) without affecting the original toxicity or antigenicity. All endotoxin solutions, for injection into the mice, were made up in pyrogen-free water (water for parenterals, Abbott Laboratories, North Chicago, Ill.).

Antisera.—Control hemolysin antisera were obtained from mice injected either intraperitoneally or intravenously with the standard antigen dose. The sera from the experimental animals were obtained after single sublethal doses of endotoxin given at various times and in different amounts in conjunction with the antigen as will be described below. Collections of the sera, pooled from brachial bleedings of three mice, were made over a period of 2 wk after immunization as will also be outlined below. The sera were stored at −20°C until used.

Antibody Determination.—Quantitative titrations of anti-sheep red cell hemolysin present in decomplemented mouse antisera (56°C for ½ hr) were performed by the method of Taliaferro (13), and the resulting antibody titer ($\log_{10} K$) expressed as the logarithm of the number of 50% anti-sheep red blood cell hemolysin units per milliliter of undiluted mouse serum. A control titration of a standard hemolysin preparation was included with each set of determinations. The use of an instrument[1] designed for the rapid and accurate delivery of small volumes permitted these estimations to be run in a total volume of 2.5 ml.

EXPERIMENTAL

To study the effect of bacterial endotoxins on the production of primary antibody, serum hemolysin titers of mice injected with the *Salmonella* endotoxin and sheep erythrocytes were compared with those of control mice given the antigen alone. The employment of sheep cells as antigen assured a rapid uptake of the injected particulate by the reticuloendothelial system (14), a prompt stimulation of antibody production, and also provided the means for a quantitative estimation of antibody in the serum (13). Further, the use of mice highly resistant to the toxicity of bacterial endotoxins

[1] Nils Jernberg, the Instrument Shop, The Rockefeller University.

(11) permitted a more comprehensive evaluation of the action of these lipopolysaccharides on processes involved in antibody synthesis, since observations were obscured but little by any harmful physiological side effects (15).

The effect of bacterial endotoxin on the primary serum hemolysin response elicited by a single injection of sheep cells was investigated by varying the dose of endotoxin and the time of its administration relative to that of the antigen. For this purpose, 15 groups, each of 27 experimental mice, received an intraperitoneal injection of the standard antigen dose of 2×10^7 sheep red blood cells on day 0. Mice in five of these groups were given a single intraperitoneal injection of 1 μg of bacterial endotoxin, each group being injected at a different time interval relative to the sheep cell injection, either 2 days or 1 day before it (called day -2 or day -1), with it (on day 0), or 1 or 2 days after it (called day $+1$ or day $+2$). Of the remaining antigen-injected mice, five groups were treated in an analogous fashion with 10 μg of endotoxin, and the last five groups with 50 μg of the lipopolysaccharide. The resulting 15 groups of mice constituted experimental series I. In order to ascertain the importance of the route employed for both endotoxin and antigen, three additional series of experiments were prepared by varying the injection route as follows: series II—endotoxin intravenously, antigen intraperitoneally; series III—endotoxin intraperitoneally, antigen intravenously; and series IV—endotoxin intravenously, antigen also intravenously. In addition, a group of 27 control mice received single standard doses of the antigen intravenously while another was given the same doses intraperitoneally. Sera obtained from the pooled bleedings of three experimental and three control animals 3, 4, 5, 6, 8, 9, 10, 12, and 14 days after the injection of antigen were titered for their content of anti-sheep red cell hemolysin (13) as outlined in the section on Materials and Methods. Sera of untreated mice, as well as those obtained from mice injected with endotoxin alone, contained no detectable amounts of anti-sheep hemolysin.

Antibody production by mice (*series I*) given *both endotoxin and antigen intraperitoneally*, and by controls injected with antigen only, is represented in Fig. 1. As can be seen, hemolysin synthesis was inhibited either partially or completely when any of the three doses of endotoxin was given before the injection of sheep cells (Fig. 1: day -1, day -2). From the responses of animals treated on day -2, the degree of inhibition can be correlated directly with the amount of injected endotoxin. A dose of 50 μg totally inhibited the hemolysin response to sheep cells, while the 10 and 1 μg doses were active to a correspondingly lesser extent. In addition, antibody synthesis appeared to be inhibited to a greater extent after giving endotoxin on day -2 rather than on day -1. On the other hand, an enhancement of hemolysin production resulted from the simultaneous injection of either the 10 or 50 μg dose of endotoxin and of sheep red blood cells (Fig. 1: day 0), while mice that received it on days after they got the antigen responded with a production of serum hemolysins similar to that of the antigen-injected controls (Fig. 1: day $+1$, day $+2$).

Groups of mice (*series II*) *injected intravenously* with 1 μg, 10 μg, or 50 μg of *endotoxin* at the specified time intervals relative to an *intraperitoneal injection* of the standard dose of 2×10^7 sheep cells elicited the hemolysin responses illustrated in Fig. 2. Under these conditions, endotoxin given before antigen produced an even greater inhibition of antibody formation (Fig. 2: day -1, day -2) than that observed in series I (Fig. 1: day -1, day -2). This is evident by the complete absence of an

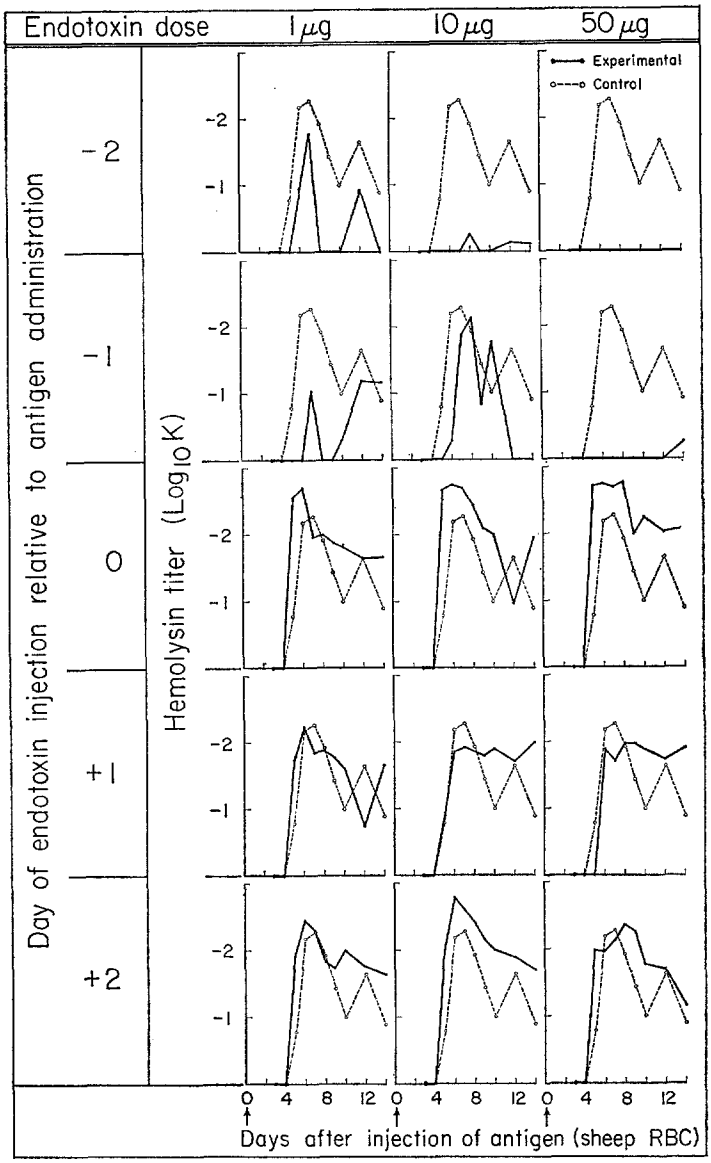

FIG. 1. The effect of the dose and time of a single injection of *S. typhosa* endotoxin on the primary hemolysin response to 2×10^7 sheep red blood cells. A comparison of serum antibody production in endotoxin-treated mice of series I (endotoxin given i.p., antigen given i.p.) and of that in controls (antigen given i.p.).

In this figure, as in Figs. 2–5, the heavy continuous lines depict the hemolysin formation by the experimental mice, those given endotoxin and antigen. The faint dotted lines represent the hemolysin formation by the control mice, those given antigen only. The antibody titer ($\log_{10} K$) is expressed as the logarithm of the number of 50% anti-sheep red blood cell hemolysin units per ml of undiluted mouse serum.

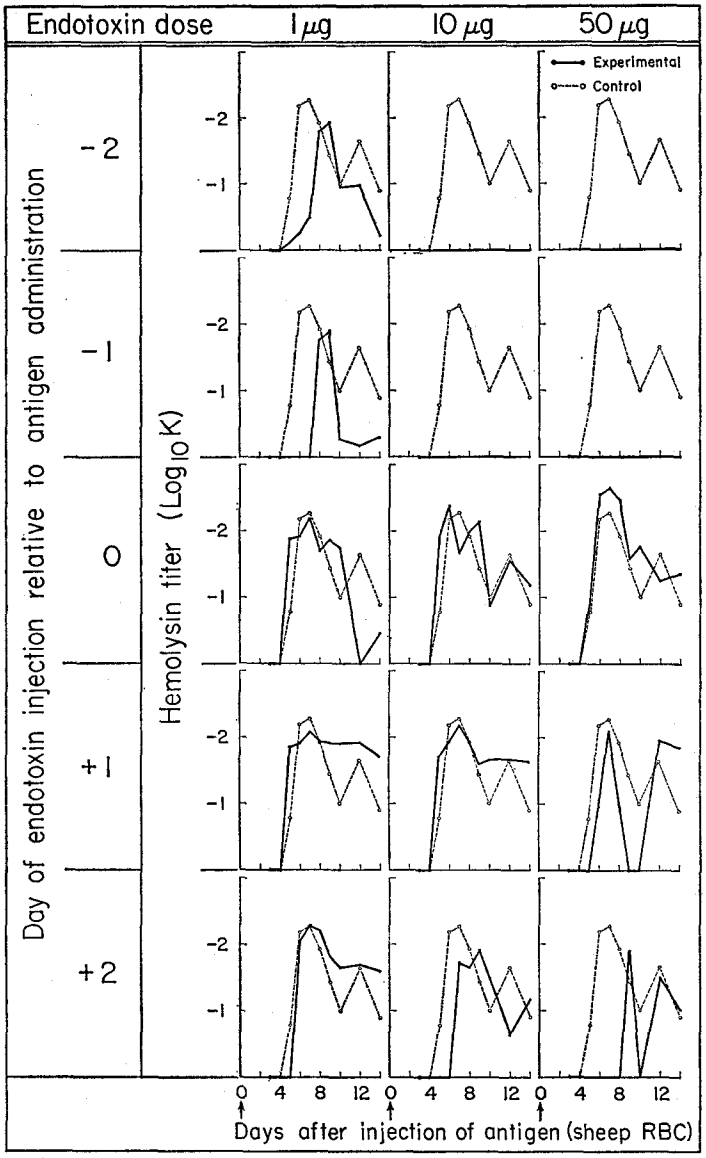

FIG. 2. The effect of the dose and time of a single injection of *S. typhosa* endotoxin on the primary hemolysin response to 2×10^7 sheep red blood cells. A comparison of serum antibody production in endotoxin-treated mice of series II (endotoxin given i.v., antigen given i.p.) and of that in controls (antigen given i.p.).

immune response in mice of series II treated with either of the two larger doses of endotoxin on either the 1st or 2nd day before antigen. The extent of the suppressive effect induced by these doses was investigated further by injecting the substance intravenously either 4 or 7 days before antigen. Under these conditions it was found, but not shown in the figures, that these levels of endotoxin still markedly inhibited the hemolysin response to an intraperitoneal injection of the standard dose of antigen. In contrast, as shown in Fig. 2, the normal production of serum hemolysin evoked by sheep cells was not affected to any great extent when any of the three endotoxin doses were given with antigen on day 0. Similar results were obtained when a dose of 1 or 10 μg was given one day after the antigen injection (Fig. 2: day +1), but when given 1 day later (day +2) these amounts partially inhibited the immune response. A 50 μg dose at these times resulted in a great suppression of hemolysin production which, though not as pronounced as on day −2 and −1, was evidenced by the considerable delay in the first appearance of antibody as well as a decrease in the total production of immunoglobulins. The data in the last two rows of Fig. 2 also demonstrate the direct proportionality of the magnitude of these inhibitions to the dose of endotoxin employed.

The intraperitoneal injection of endotoxin into mice (series III) at specified times relative to intravenous challenge with sheep red cells resulted in the hemolysin responses shown in Fig. 3. The appearance in some experiments of heightened serum titers on the 5th and 6th days of the response (Fig. 3: day −2, day −1, day 0) represents a higher initial rate of antibody production in animals given the particular doses of the lipopolysaccharide before or with the injection of antigen compared to the rate in controls injected with sheep red blood cells. Since this maximal stimulation was evident only after giving 1 μg of endotoxin on day −2, or 10 μg on day −1, or 50 μg on day 0, its occurrence was clearly dependent not only upon the amount but also on the time of the injections. In addition, the largest experimental dose of endotoxin greatly prolonged the much enhanced hemolysin response when given one day after antigen (on day +1), but, if administered a day later (on day +2), its action was opposite as seen by the resulting inhibition of antibody synthesis. As can be noted, lower doses of endotoxin on either day 0, +1, or +2 had no appreciable effects on serum hemolysin production.

The data presented in Fig. 4 represent the anti-sheep hemolysin responses resulting from the *intravenous injections of both endotoxin and antigen* to the mice of *series IV*. As can be seen (Fig. 4: day −2, day −1), the lowest dose of endotoxin before the antigen had either no effect or slightly enhanced the production of serum hemolysins, while 10 or 50 μg caused considerable inhibition of the antibody response and even complete suppression (50 μg, day −1) under identical experimental conditions. In contrast, all three doses of endotoxin greatly enhanced the production of antibody in mice when given at the same time or 1 day after the standard antigen injection (Fig. 4: day 0, day +1). These hemolysin responses, which were accompanied by a shortened induction period as well as a heightened and prolonged production of antibody, represent the greatest augmentation of the primary hemolysin response to 2×10^7 sheep red blood cells obtained in the study of all four experimental series. However, as seen in the last row of Fig. 4, endotoxin 2 days after the standard antigen injection inhibited

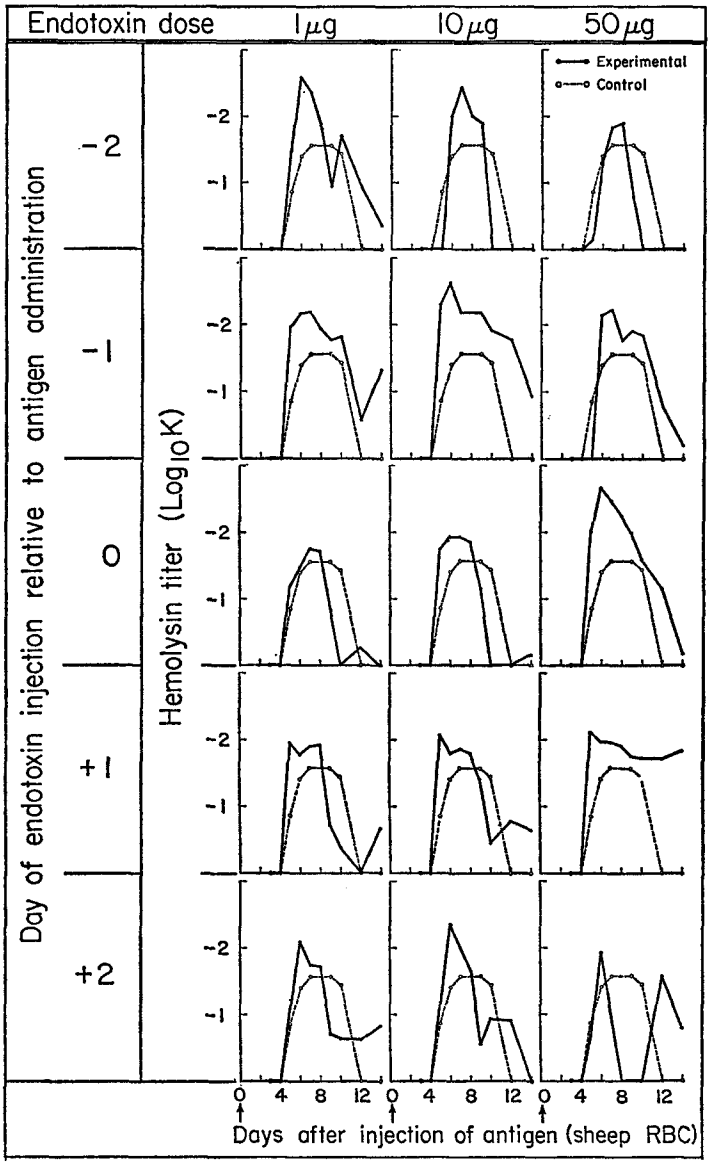

FIG. 3. The effect of the dose and time of a single injection of *S. typhosa* endotoxin on the primary hemolysin response to 2×10^7 sheep red blood cells. A comparison of serum antibody production in endotoxin-treated mice of series III (endotoxin given i.p., antigen given i.v.) and of that in controls (antigen given i.v.).

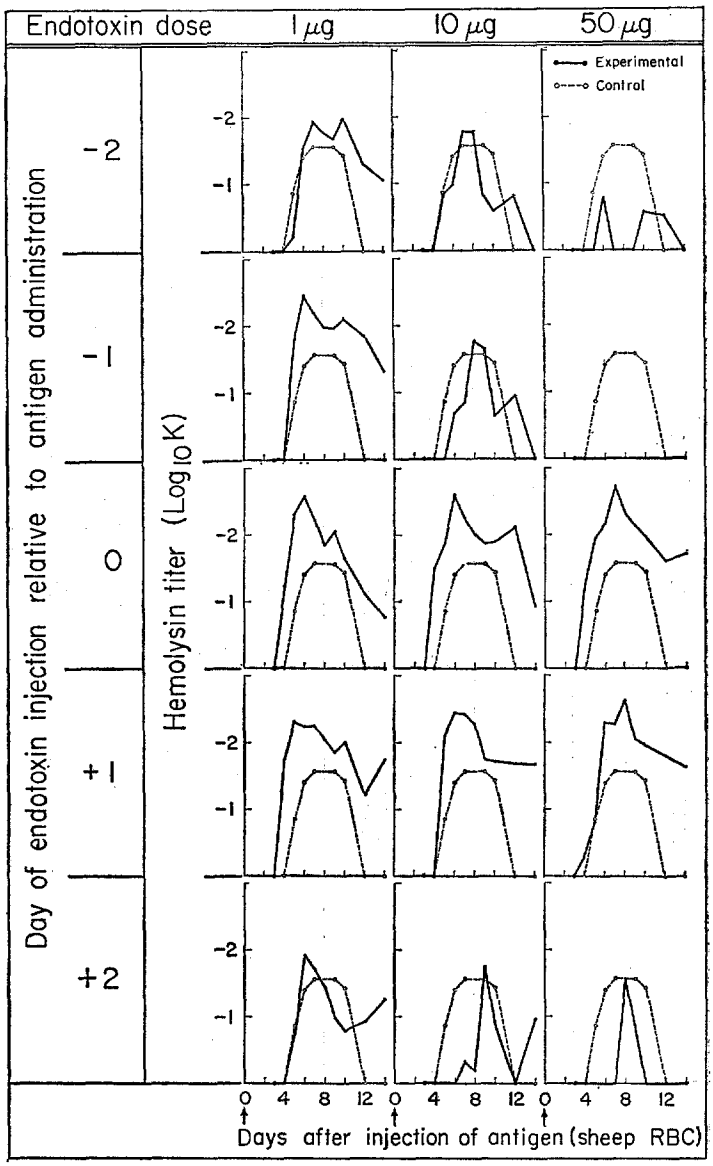

FIG. 4. The effect of the dose and time of a single injection of *S. typhosa* endotoxin on the primary hemolysin response to 2×10^7 sheep red blood cells. A comparison of serum antibody production in endotoxin-treated mice of series IV (endotoxin given i.v., antigen given i.v.) and of that in controls (antigen given i.v.).

the normal production of antibody, the magnitude of this effect correlating directly with the dose employed.

In an attempt to relate the toxicity and chemical composition of bacterial endotoxins to their suppressive and adjuvant effects on antibody production, a study was made of the changes in the immune responses of experimental mice to the standard dose of sheep erythrocytes as influenced by injections of four dissimilar *E. coli* lipopolysaccharide preparations, differing in their toxic properties as well as in their content of

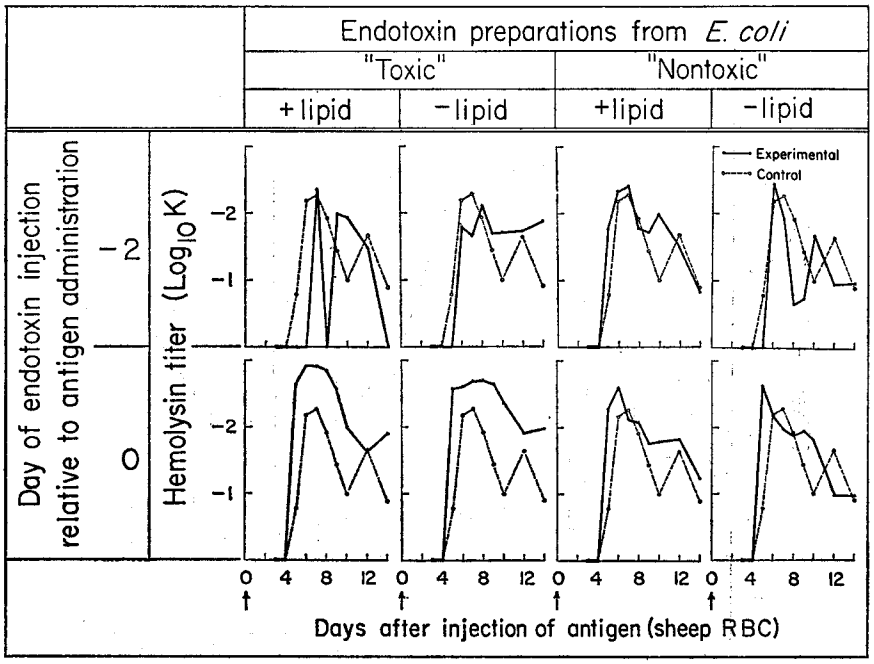

FIG. 5. The effect of the time of a single injection of 50 μg of different *E. coli* endotoxin preparations (see Materials and Methods) on the primary hemolysin response to 2×10^7 sheep red blood cells. A comparison of serum antibody production in endotoxin-treated mice (endotoxin given i.v., antigen given i.p.) and of that in controls (antigen given i.p.).

"bound" lipid (see the section on Materials and Methods). Toward this end, mice were intravenously injected with 50 μg of each endotoxin preparation either 2 days before, or on the same day that antigen was given intraperitoneally. The resulting serum hemolysin responses were analyzed as described above.

The manner in which these different preparations affected the primary hemolysin response is illustrated in Fig. 5. As can be seen "toxic" preparations, when given before or with antigen, changed the course of the immune response. The toxic preparation, containing bound lipid, partially inhibited the normal production of antibody when the dose preceded the antigen, while the toxic product of the same material, with this lipid component removed, had no effect under these experimental conditions. On the other hand, either of these materials, given together with the antigen on day

0 produced a marked augmentation of the normal antibody response. These findings are in contrast with those obtained with the two "nontoxic" endotoxins since, as shown in Fig. 5, the latter had no observable effect on the production of hemolysins in mice.

To substantiate further the inhibitory as well as the enhancing effects of bacterial endotoxin on the production of anti-sheep hemolysins, random bred albino mice,

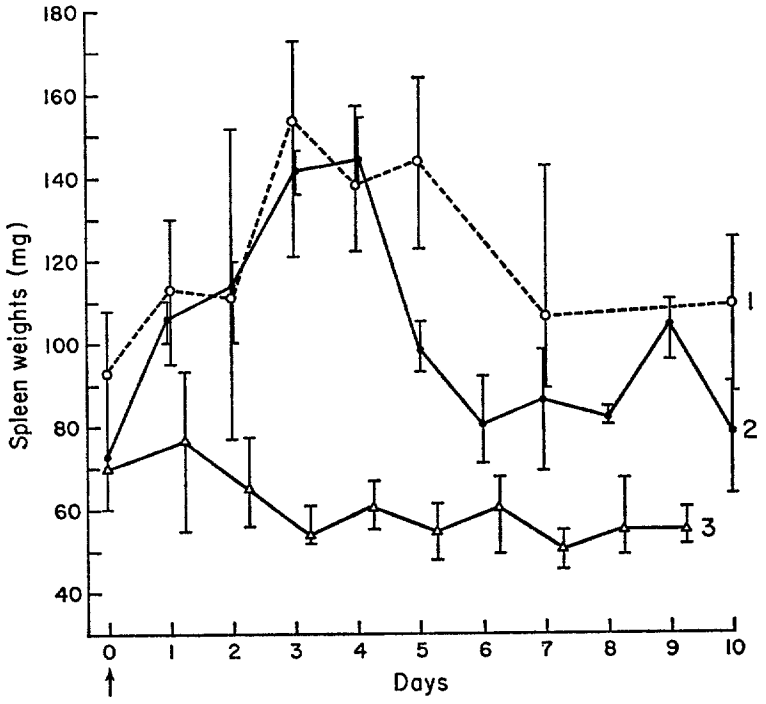

FIG. 6. The increase in the weight of the mouse spleen after i.p. injection of 50 μg of *S. typhosa* endotoxin. Spleen weights after endotoxin (curve 1), after endotoxin and 2 $\times$ 10^7 sheep red blood cells (curve 2), and after sheep erythrocytes (curve 3). The points represent the average weights of three to five organs, the ranges of which are given by the neighboring vertical lines.

Princeton strain,[2] were treated in a manner similar to that previously described in this report. Each of the three doses of lipopolysaccharide *S. typhosa* was given intraperitoneally either 2 days before or on the same day as the intraperitoneal injection of the standard dose of antigen. The results obtained with mice of the Princeton strain were like those already described above in series I with mice of the Nelson-Collins strain. In both instances, the injection of endotoxin before the antigen inhibited the production of serum antibody, whereas with it the immune response was enhanced.

[2] These mice were made available through the generosity of Dr. John B. Nelson of The Rockefeller University.

The weights of two lymphoid organs from both experimental and control mice showed that endotoxin caused an increase in the size of the spleen and a decrease in the weight of the thymus. These effects were directly proportional to the dosage of the lipopolysaccharide, the thymus being more noticeably affected by this treatment. A graphic representation of the weights of spleens after the single intraperitoneal injections of 50 μg (Fig. 6: curve 1) demonstrates the early rapid increase in the weight of this organ, the maintenance of maximum values from the 3rd to the 5th day, and the gradual return of the normal weight range by the 7th day. As can be seen by curve 2, the simultaneous administration of 2×10^7 sheep red cells with the endotoxin did not significantly alter this picture. On the other hand, thymus glands from the same animals showed, as also reported by others (16), a decisive drop in weight from their normal average 100 mg to only 30–40 mg on the 2nd day. This low range endured through day 7 and then gradually increased until the weights reached normal levels on approximately the 11th day. The standard erythrocyte injection had no effect upon the weight of the spleen (curve 3) or on that of the thymus.

DISCUSSION

The standard dose of 2×10^7 sheep erythrocytes employed in this study led to an appreciable but not maximal production of serum hemolysin (see dashed lines of Figs. 1–4) while avoiding an accumulation of excess antigen in the various phagocytic tissues. Although the spleen is recognized as the major site of hemolysin production after an intravenous injection of this particulate antigen (17), the different response curves resulting from intravenous and intraperitoneal injections of foreign erythrocytes (compare dashed lines of Figs. 3 and 4 with those of Figs. 1 and 2) support the contention that, in addition to this organ, the lymphoid tissues adjacent to or within the peritoneal cavity (18, 19) may also function as producers of antibody after sheep red blood cells have been given intraperitoneally. The manner in which various doses of endotoxin affect the sites of hemolysin formation was studied to advantage by observing, for a period of 2 wk, the changes in the serum content of immunoglobulins with a relatively short half-life (20). Bacterial endotoxins cause, in addition to an array of physiological alternations, a "stress" reaction in lymphatic tissues (21), an enhancement of the phagocytic capacity of the reticuloendothelial system (2), and when given intraperitoneally, a local accumulation of phagocytes in the peritoneal cavity (22).

A summary of our findings, obtained by comparing the immune reactions occurring in endotoxin-treated mice of series I–IV with those in antigen injected controls (see Figs. 1–4), is presented in Table I. Results comparable to those of experimental series IV (lower row of Table I) have also been obtained in rats by similarly timed injections of formalin-killed *Salmonella typhimurium* (7).

Endotoxin Given Simultaneously with Antigen on Day 0.—Most often under these circumstances, endotoxin acted as an adjuvant and enhanced the production of serum hemolysin to the standard antigen injection (Table I: Day

0). Since 1 and 10 μg doses in series I and IV had this effect only when given by the same route as the antigen (compare series I and IV with series II and III), the enhancements obtained in day 0 experiments are presumed to have resulted from the simultaneous uptake of endotoxin and antigen, possibly as

TABLE I

The Effect of S. typhosa Endotoxin on the Primary Hemolysin Response in Mice

Experimental series	Antigen* injection route	Endotoxin injections						
		Route	Dose	Time (days)‡				
				−2	−1	0	+1	+2
			μg	§				
I	i.p.	i.p.	1	−	−	+	0	+
			10	−−	−	++	+	++
			50	−−	−−	++	+	0
II	i.p.	i.v.	1	−	−	0	+	0
			10	−−	−−	0	0	−
			50	−−	−−	+	−	−
III	i.v.	i.p.	1	+	+	0	0	0
			10	+	++	0	0	0
			50	−	+	++	++	−
IV	i.v.	i.v.	1	0	+	++	++	0
			10	−	−	++	+	−
			50	−−	−−	++	+	−

* 2×10^7 sheep red blood cells in 0.1 ml was given on day 0.

‡ The figures indicate the day of endotoxin injection in relation to the administration of antigen (day 0). Negative numbers denote days before, positive numbers, days after the injection of antigen.

§ The effects of the bacterial endotoxin on the hemolysin response were estimated from the data in Figs. 1–4, and designated as follows: −−, total inhibition; −, partial inhibition; 0, no change; +, slight enhancement; ++, maximum enhancement.

endotoxin-coated erythrocytes, by those phagocytes associated with the immune response. The consistent adjuvant effects of 50 μg of endotoxin in all experimental series (Table I: day 0; series I–IV) may also be attributed to a concomitant ingestion with the particulate antigen, made possible by the sustained presence of endotoxin in the bloodstream after injecting large doses (23). In contrast, the ineffectiveness of the 1 and 10 μg doses, when given by routes alternate to those employed for the antigen in series II and III, points to an inactivation or an efficient removal of small amounts of free lipopolysaccharide by cells not committed

to the immune response. This is supported further by the contrasting effects, exerted by the same small doses on the splenic processes responding to intravenously injected sheep red blood cells, illustrated by series III and IV in Table I. From the inactivity of the intraperitoneal injections in the first instance, and the enhancements of the intravenous treatments in the second, it is reasonable to conclude that only a fraction of endotoxin from the peritoneal cavity gains direct access to the bloodstream in a form comparable in activity to material put directly into the circulation. In accordance with this view, it may also be noted (Table I) that, when given intraperitoneally, only 50 μg of endotoxin (series III) is able to enhance the production of hemolysin to the same degree as 1 μg of the lipopolysaccharide when presented by the intravenous route (series IV). It is of interest that none of the endotoxin doses given on day 0 suppress the production of hemolysins.

Endotoxin Given before the Antigen (Day −2 and −1).—In contrast to the stimulating effects of endotoxin given on day 0, single injections of the bacterial lipopolysaccharide prior to sheep red cells inhibited the production of hemolysin in most experiments except those of series III (Table I: day −2, day −1). Given intravenously, endotoxin inhibited immune responses evoked by both intravenous as well as by intraperitoneal antigen injections (series II and IV), suggesting that 24 hr after its injection into the bloodstream endotoxin has directly impaired the functional activity of antibody production sites located primarily in the spleen. In contrast, when given intraperitoneally, the lipopolysaccharide inhibited only those hemolysins produced by intraperitoneally presented sheep cells (series I) and actually enhanced significantly the amounts of antibody elicited by intravenous injections of the antigen (series III). These observations indicate that, under these conditions, the lipopolysaccharide has probably acted by different mechanisms. Thus the inhibited reactions in series I may be explained by the inaccessibility of the sheep cell antigen to splenic antibody-producing sites as a result of the retention and destruction of the particulate antigen by the large numbers of phagocytes in the peritoneal cavity after the intraperitoneal inoculation of endotoxin (22), while the enhanced production of antibody in series III implies that, after 1 or 2 days, in addition to stimulating peritoneal cell exudates, endotoxin or its products derived from peritoneal action, can also activate the spleen either directly or indirectly to higher antibody formation.

Endotoxin Given 1 and 2 Days after Antigen (Day +1 and +2).—The fewer as well as weaker enhancements produced by injecting endotoxin 1 day after the standard dose of sheep red cells (Table I: day +1; series I and IV) indicate that lipopolysaccharides are capable of exerting only a limited stimulatory action on the spleen during this early period of the hemolysin response. The stimulatory effects of the low doses, when given on day + 1 and +2 in series I (see Table I), are undoubtedly the consequence of having employed

the intraperitoneal route for antigen injection (note the complete absence of any effect on hemolysin production in the parallel experiments of series III) and possibly represent an additional stimulation of extrasplenic sites of hemolysin synthesis previously activated by the intraperitoneal presence of sheep erythrocytes (19). The inactivity of the smaller doses given intraperitoneally in series III (Table I: day +1 and +2) can be ascribed, as previously discussed for day 0 experiments, to an inadequate effective concentration of the substance at antibody-producing sites. This view is supported by the fact that on day +1 both the intraperitoneal administration of 50 μg of endotoxin (series III) as well as the intravenous injection of the 1 or 10 μg amounts (series IV) greatly enhanced the immune responses to the intravenously injected antigen.

The previous discussions of the findings in Table I emphasize the importance of the route of injection in evaluating the action of bacterial lipopolysaccharides on the production of hemolysin in mice. Similarly, the method of giving endotoxin is known to affect appreciably the compound's activity as a toxin (23), as a modifier of the phagocytic efficiency of the reticuloendothelial system (24, 25), and as a protector against infection (25–27).

The direct influence of endotoxin on splenic antibody formation can be evaluated by studying the effects of a small intravenous dose of this compound on the production of hemolysin elicited by an intravenous injection of sheep erythrocytes. Results of such experiments in series IV, presented in the bottom section of Table I, show that 1 μg of endotoxin exerted a strong adjuvant action when given 1 day before, with, or 1 day after the injection of sheep red cells (day −1, 0, and +1), but was without effect either 2 days before or 2 days after the antigen (day −2 and +2). From this it may be concluded that within a 3 day period this bacterial endotoxin maintains a direct activation of splenic processes concerned with the immune mechanism for approximately 24 hr. These findings, as well as those of histological investigations (9) demonstrating the increased number of large pyroninophilic cells in lymphoid tissues in endotoxin-stimulated mice during this period, support the hypothesis (4) that, as adjuvants, bacterial endotoxins activate the early phases of the immune response.

It has previously been established that bacterial endotoxins, when given with or shortly after a protein antigen, enhance the immune response in a number of animal species (4, 5, 28). Specifically, Merritt and Johnson (5) have shown that mice, treated intraperitoneally with a single 50 μg injection of endotoxin within a period of 7 days before to 6 days after a similar injection of bovine gamma globulin, respond with a greatly increased production of antibody and a striking decrease in the length of the induction period. The results of our present studies with mice are in general agreement with these findings. As shown in Table I, the production of hemolysin to sheep red cells

is enhanced when 1, 10, and 50 μg doses of endotoxin are given with or shortly after the particulate antigen (series I and IV) or before it (series III). Shortened induction periods are also obtained (see 3rd and 4th rows of Fig. 4), but accompany only those heightened responses resulting from the injection of both red blood cells and endotoxin intravenously (series IV).

On the other hand, it is also evident from Table I that sublethal doses of endotoxin can inhibit the primary immune response in mice. The complete or partial suppression of serum hemolysin production (Table I and Figs. 1, 2, and 4) is most pronounced when endotoxin is given before the sheep cells (day −2, −1; series I, II, and IV) and, as previously discussed, may result from the retaining action of peritoneal phagocytes on antigen in the peritoneal cavity (series I) or from a cytotoxic action on antibody forming tissues (series II and IV). The latter hypothesis is supported by the finding that a single injection of endotoxin, even when given 7 days prior to antigen, effectively inhibits the production of antibody, and also by histological observations (9) revealing a conspicuous lack of cellular changes characteristic of a primary response to sheep red cells in the lymphoid tissues of the experimentally inhibited mice. Whether these inhibitory effects are comparable to the immunosuppressive action of potent metabolic inhibitors (29), as suggested by a report of the inhibitions by 6-mercaptopurine of an immune response to foreign erythrocytes in mice (30), is still to be established. Although lipopolysaccharides are good antigens and induce the production of specific antibodies (31), it is unlikely that the marked decrease of anti-sheep red cell hemolysin production evoked by single injections of relatively small amounts of these compounds can be explained by the immunological phenomena of antigen competition (32).

The enhancements and inhibitions of hemolysin production in two unrelated strains of mice by the proper administration of *S. typhosa* endotoxin described herein, and the similar effects in rats given timed doses of formalin-killed *S. thyphimurium* reported by Simic et al. (7), establish unequivocally the marked variability of the action of endotoxin on hemolysin formation. These findings also illustrate the similar manner by which bound and free endotoxins influence the course of the primary hemolysin response. Results ranging from complete suppression to marked enhancement of antibody synthesis to protein antigens have also been obtained by manipulating the dosage and the time of administration of the antimetabolites 6-mercaptopurine (33), and 5-fluoro-2′-deoxyuridine (34). However, since these effects resulted from the administration of these compounds under circumstances which differed greatly from those employed with endotoxins, one is reluctant to employ the mechanism of action of the relatively large doses of antimetabolites to explain the activity of small amounts of lipopolysaccharides. The difference between the effect of endotoxin on antibody production to sheep red cells on one hand, and its influence on the

response to foreign globulins (5) on the other, strongly suggests that particulate and soluble antigens are handled differently during the early stages of the immune response. This proposal is supported further by reported differences in the cellular responses of lymphoid centers to various types of antigens (35). The suppression of endotoxin of the immune response to actinophage in mice reported by Bradley and Watson (8), although agreeing only partially with the results of our investigations, constitutes additional supporting evidence for the inhibitory action of these compounds on immune processes stimulated by particulate antigens. In view of the enhancing and suppressive effects obtained in the present studies with sheep erythrocytes, the absence of any adjuvant effects in the investigations with actinophage may possibly reflect the well-known difference between the relative susceptibility of the two particulate antigens to phagocytic degradation.

The variability in the biological activity of bacterial lipopolysaccharides has, in many instances, been ascribed to the methods employed for their isolation and purification (36). Results of our investigations with a number of endotoxin preparations from *E. coli* demonstrate that certain lipid-containing preparations both enhanced and inhibited the production of antibody in a manner similar to the commercial preparation of *S. typhosa* lipopolysaccharide. It is of interest that both endotoxins were toxic and antigenic and had been isolated by similar Boivin extractions of bacterial cell walls; the one from *S. typhosa* by direct trichloroacetic acid treatment (37),[3] the other by the same method after preliminary solvent extraction (see Materials and Methods). The finding that removal of loosely bound lipid from the toxic *E. coli* endotoxin resulted in a loss of the inhibitory action and the retention of the original capacity for enhancement (see Fig. 5) parallels the report that chemical treatments abolished several biological properties of lipopolysaccharides without affecting their adjuvant action (38). Since the administration of antigenic (12) nontoxic *E. coli* preparations did not affect the normal course of hemolysin production, it may be stated that the inhibitory activity of endotoxin is possibly linked with loosely bound lipid components in toxic bacterial preparations, while, in agreement with others (39, 40), the enhancing action appears to be associated with that region of the endotoxin molecule responsible for its toxic properties.

The initial distribution of particulate and soluble antigenic material in macrophages of the reticuloendothelial system (1, 14) is thought to be essential for the induction of the primary antibody response (41). This hypothesis is supported by evidence that materials which stimulate the phagocytic activity of this system, such as zymosan (42), glucan (43), and the Calmette-Guérin bacillus (44), also enhance antibody formation to sheep red blood cells, while inhibitors of reticuloendothelial phagocytic function, such as cholesterol

[3] Difco Supplementary Literature. 1964. Difco Laboratories, Detroit.

oleate (45) and methyl palmitate (46), depress the immune response to foreign erythrocytes. It is also known that a large portion of bacterial endotoxin, when injected intravenously, is removed from the circulation within 1 hr by cells of the system (47, 48) and detoxified by the spleen and liver (49). This results in an initial depression and then, after approximately 48 hr, an enhancement of the phagocytic capacity of the reticuloendothelial system, as shown by studies of the blood clearance rate of colloids (2, 3) and of the activity (50) and metabolism (51) of phagocytic cells. Accordingly, the inhibitions of hemolysin formation by single injections of a bacterial endotoxin, given prior to antigen, can be ascribed to the extended period of hyperactivity of the reticuloendothelial system known to be present in mice after lipopolysaccharide treatments (47), while the enhancements, generally produced by the simultaneous or later injections of endotoxin, can result from suppression of phagocytic activity. Inasmuch as one or more of a variety of physiological changes induced by lipopolysaccharides (15) might also influence the formation of antibodies, it is difficult to correlate the inhibitions and enhancements of hemolysin production reported herein with changes in phagocytic activity. It remains to be seen whether immediate effects, such as the transient granulocytopenia (52) associated with an increased susceptibility to microbial infection (53) and the increased synthesis of proteins of the serum, lymph nodes, spleen, and adrenal glands (54) also influence the formation of hemolysins. It has also been reported that endotoxins elicit a transient multiplication of both bactericidin- and hemolysin-forming cells in the spleen (30, 55). In reference to the latter, hemolysins to sheep were not detectable in the serum of mice given single injections of endotoxin.

SUMMARY

The manner in which a single injection of *S. typhosa* endotoxin effects the primary hemolysin response to sheep erythrocytes in the mouse has been shown to depend on the dosage, route, and time of administration of the endotoxin, as well as on the route employed for the injection of antigen. The normal production of antibody, following an intravenous or an intraperitoneal injection of red blood cells, is suppressed if the bacterial lipopolysaccharide is given before and by the same route as the antigen. The response to an intraperitoneal injection of sheep red cells is also inhibited if preceded by an intravenous injection of endotoxin. By contrast, hemolysin formation to intravenous antigen is enhanced considerably by a previous intraperitoneal injection of endotoxin, and the response both to intravenous and to intraperitoneal injections of the antigen increases if the endotoxin is given by the same route either simultaneously or shortly after the foreign red cells. These findings are discussed in regard to the physiological action of bacterial endotoxins and the early events in antibody formation.

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Note Added.—The inhibitory action of endotoxin on antibody formation to a soluble antigen, bovine gamma globulin, has recently been reported. Johnson, A. G., A. Jacobs, G. Abrams, and K. Merritt. 1967. Comparative changes in the mouse spleen during immuno-stimulation or immuno-suppression. *In* Germinal Centers in Immune Responses. H. Cottier, N. Odortchenko, R. Schindler, and C. C. Congdon, editors, University of Bern. Springer-Verlag, New York. 234.

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